REMARKS

No new matter is introduced by the amendments.

The addition of the recitation "elicits protective antibodies" in claim 1 and claim 16 is supported throughout the specification. For example, at page 4, lines 29-31: "An aspect of the invention is a method of eliciting the production of antibodies in mammals using the β-propionamide-linked polysaccharide-protein conjugates that protect the mammals against infection or disease." Also, the specification discloses data that the claimed conjugates do elicit protective antibodies at Tables 5-7, Opsonophagocytic assays (OP).

The replacement of the recitation "comprising an N-propionated polysaccharide or N-propionated oligosaccharide" with "comprise an N-propionated saccharide" does not introduce new matter. This use of the term "saccharide" is used to simply reflect that claimed conjugates may comprise either oligosaccharides or polysaccharides. Support is provided throughout the specification. For example, at page 7, Section A, "Preparation of the N-acryloylated polysaccharides", the specification states that the starting material may be polysaccharides or oligosaccharides: "Polysaccharide or oligosaccharide may be obtained using base hydrolysis or enzymatic hydrolysis..." (page 7, lines 8-9). Support for the addition of "and wherein the N-propionated saccharide is de-N-acetylated and N-acryloylated at the de-N-acetylated terminus" in claim 1, is found throughout the specification, for example, at page 10, lines 6-14.

The addition of the recitations "at a de-N-acetylated terminus" and "coupling at a β-position of a propionate moeity" in claim 16 are supported, for example, at page 10, lines 6-14 and at Example 2. These amendments were made in order to more clearly describe the claimed invention and add no new matter.

The addition of the term "obtained" to claims 3, 4, 5, 17, and 37 was made in order to respond to the Examiner's concern that the term "derived" is indefinite. Applicants disagree with the Examiner's contention concerning the recitation of "derived" which applicants believe properly and definitely describes applicants' invention. The use of "derived" is appropriate because the polysaccharides which are obtained from cells for use in the conjugates are then subjected to modification by being N-acryloylated at de-N-acetylated terminal groups prior to conjugation to protein. Support for the addition of "obtained" is found at page 7, line 8.

Other changes to the claims were made in response to rejections and objections

made by the Examiner. Detailed responses to these rejections and objections are stated in this Response below. No new matter has been added by these amendments.

The changes to the specification only reflect changes to comply with stylistic norms. Entry of the amendments are respectfully requested.

Response to Specification Objection (Examiner's Action #7(c)):

The specification is objected to because the Examiner contends that the claim recitations "N-propionated polysaccharide" and "N-propionated oligosaccharide" do not appear to have antecedence in the specification. Applicants respectfully disagree.

First of all, the recitations "N-propionated polysaccharide" and "N-propionated oligosaccharide" are used in the claims as originally filed, and thus these recitations are disclosed in the specification. Also, the claim recitations "N-propionated polysaccharide" and "N-propionated oligosaccharide" define the claimed invention with a reasonable degree of clarity and precision. The methods used in the instant application apply for both oligosaccharides and polysaccharides. Support is found throughout the specification. For example, at page 8, lines 25-29, under Section 2 "N-Acryloylation of the Polysaccharide" (i.e., the process of making N-propionated saccharides), the specification recites:

> The alkaline or enzymatic hydrolysis of the polysaccharide or oligosaccharide results in the removal of N-acetyl groups from sialic acid and amino sugar residues of the polysaccharides or oligosaccharides. After hydrolysis, the polysaccharide or oligosaccharide is N-acryloylated to the extent desired by using a variety of acryloylating agents.

Thus, the recitations of "polysaccharide" and "oligosaccharide" are supported for the claimed conjugates and methods.

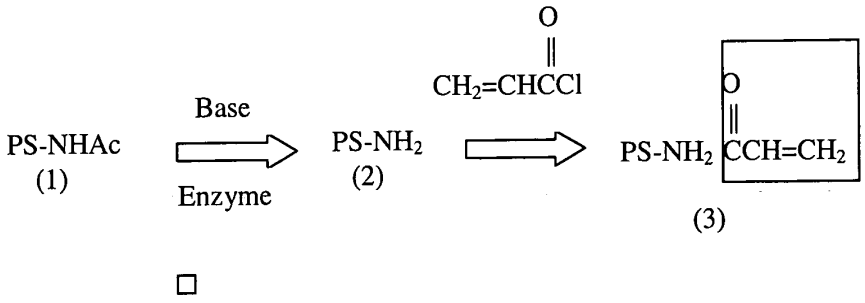
Applicants respectfully disagree with the Examiner's objection to the recitation "N-propionated". First of all, the recitation "N-propionated" is used in the claims as originally filed, and thus the recitation "N-propionated" is disclosed in the specification. Also, the recitations "N-propionated polysaccharide" and "N-propionated oligosaccharide" are defined by a claimed method of forming a immunogenic polysaccharide- or oligosaccharide-protein conjugate. For example, on page 10, lines 6-14, the instant specification states:

> In one method of forming a immunogenic polysaccharide-protein conjugate, an isolated polysaccharide (glycosaminoglycan)

> containing free amino groups or N-acyl groups (e.g. N-acetyl groups) in the sugar residues that constitute its repeating unit, is first treated hydrolyzed using base or enzyme to remove part of all of its N-acyl groups. The free amino groups are then N-acylated with an N-acryloylating reagent to form the N-acryloylated polysaccharide described above. The N-acryloylated polysaccharide is then directly coupled to protein under optimum conditions of pH, temperature and time to form an immunogenic β-propionamido-linked polysaccharide-protein conjugate.

After deacetylation, the free amino groups of a polysaccharide or oligosaccharide are re-N-acylated with an N-acryloylating reagent, as described above, to form the N-acryloylated polysaccharide. This N-acryloylated polysaccharide (oligosaccharide) is equivalently described within the art as an "N-propionated polysaccharide (oligosaccharide)" because the process of N-acryloylation forms a propionate group where the sugar residue has been de-N-acetylated. Again, the methods disclosed in the instant application may be used for <u>both</u> poly- and oligo-saccharides as the specification mentions this fact repeatedly. For example, "This invention provides the ability to produce conjugate molecules wherein the protein is linked to the polysaccharide or oligosaccharide through one or more sites on the polysaccharide or oligosaccharide." (page 9, lines 30-32).

Further support for the process of making N-propionated saccharides can be found in the specification at the paragraph spanning page 10 and page 11, and in Figure 1. Applicants have duplicated Figure 1 on the following page to diagram a claimed method of forming a immunogenic polysaccharide- or oligosaccharide-protein conjugate, so that applicants may show how a polysaccharide (and oligosaccharide) is N-propionated.



In step (1), the polysaccharide is de-acetylated by either base or enzyme, resulting in product (2).

The de-acetylated polysaccharide is then N-acryloylated, so that in (3), the polysaccharide has a propionate group (boxed region in (3)) where originally an acyl group resided (1). Thus, the process of N-acryloylation in the instant invention forms an N-propionated polysaccharide or oligosaccharide. Applicants respectfully request reconsideration and withdrawal of this ground of objection.

Response to Section 112, First Paragraph Rejection (Examiner's Action #8):

Claims 18 and 20 have been rejected because the Examiner contends that the specification does not reasonably provide enablement for a polysaccharide/oligosaccharide-protein conjugate wherein the conjugation is conducted at a pH of 7.0 and in a phosphate buffer.

Specifically, the Examiner refers to various reports in the art (Romanowska *et al.* (1994), Roy *et al.* (1990), Roy *et al.* (1991), and Pon (1992)) as support for her contention that undue experimentation would be required to conjugate an N-acryloylated polysaccharide or oligosaccharide by Michael addition at a pH of about 7.0 in a phosphate buffer medium, because the Examiner contends that the reports cited above show that such conjugation would not work. Thus, the Examiner states "This is important because there is no certainty that this type of conjugation could optimally and/or effectively be conducted at a non-alkaline pH."

The instant specification provides clear guidance as to when conditions of neutral pH should be used so that conjugation would be effective:

> In one embodiment, the method of conjugation is conducted at a pH above 9.0, preferably a pH of about 9.0 to about 10.0 for optimal reactivity of ε-free amino groups of lysine residues on the protein. In another embodiment, the method of conjugation is conducted at a neutral pH of about 7.0 for optimal reactivity of thiol (SH) groups of cysteine residues of the protein. The selection of pH for conducting the method of conjugation may be based on the number of reactive groups in a particular carrier protein. For example, a method using a protein composed of more reactive lysine residues as compared to cysteine residues is preferably conducted at a basic pH. A method of conjugation using a protein composed of more reactive cysteine residues as compared to lysine residues is preferably conducted at about a neutral pH. (page 10, lines 15-25).

The references cited by the Examiner do not mention anything about conjugation of cysteine-rich proteins to polysaccharides. Therefore, applicants respectfully assert that the Examiner's

enablement rejection is improper, since "it is incumbent upon the Patent Office, whenever a rejection on this basis [enablement] is made, to explain why it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement." (MPEP §2164.04). Applicants respectfully contend that the Examiner has not provided a basis for which to reject the truth or accuracy of the specification's guidance for the conjugation of cysteine-rich proteins at neutral pH.

As for conjugation in phosphate buffers, the Examiner's basis of enablement rejection is also unfounded. Although Romanowska *et al.* states "There was only very slow coupling in phosphate buffers," (page 101), applicants respectfully point out that there was coupling nevertheless. Accordingly, applicants respectfully request reconsideration and removal of this ground of rejection.

Response to Section 112, First Paragraph Rejection (Examiner's Action #9):

Claims 25 and 40 have been rejected under 35 U.S.C. §112, first paragraph, because the Examiner contends that the specification does not enable a pharmaceutical composition or vaccine comprising more than one vaccine component. In particular, the Examiner argues that there is no showing by the specification that combination vaccines comprising an N-propionated polysaccharide or oligosaccharide protein-conjugate would effectively elicit an optimal immune response. The Examiner relies upon two references, Barrington *et al.* (*Infect. Immun.* 61:432-438, 1993) and Corbel (*Biologicals* 22:353-360, 1994) to support her contention that the art reports "potential interference by one or more added vaccine components and suppression of antibody response to the polysaccharide or the carrier protein". Therefore the Examiner argues that undue experimentation would be required by one of ordinary skill in the art to practice the invention as claimed in claims 25 and 40.

Applicants respectfully disagree with the grounds of this rejection. The Examiner asserts that combination vaccines may lead to epitope suppression of anti-polysaccharide responses and therefore claims 25 and 40 would require undue experimentation to practice the invention as claimed. However, this assertion is highly debated in the art, and that combination vaccines have been used repeatedly in the art with great success. For example, Pichichero *et al.* (*J. Infect. Dis.* (1999), 180:1390-3; copy enclosed) reports data that would argue against the

contention that epitope suppression occurs with combination vaccines:

> There have been concerns about interference with Hib vaccine responses, manifested as a decrease in the anti-Hib-PS antibody level, when Hib and DTaP vaccines are combined. We recently studied a DTaP-PRP-T-HB combination vaccine and found evidence that immunologic memory was induced even in infants with lower (<1.0 μg/mL) and even undetectable (<0.10 μg/mL) postprimary anti-Hib-PS antibody levels. Thus, we suggested that the combination vaccine primed the infant immune system for anamnestic anti-Hib-PS antibody responses. Affinity maturation is another major feature of immunologic memory. Here, we showed that DTaP-PRP-T-HB vaccines elicit a high avidity IgG antibody against the Hib PS antigen. Unexpectedly, avidity increased most significantly in the 3-7 months after primary DTaP-PRP-T-HB vaccination, with a marginal further rise after a CRM_{197}-OS booster. (page 1392, first paragraph under Discussion section).

In addition, Goldblatt *et al.* (*J. Infect. Dis.* (1999), 180:538-41; copy enclosed), reports that despite reduced immunogenicity, DTaP-Hib combination vaccines appear to prime for immunologic memory. Thus, the article by N. Halsey, ("Combination Vaccines: Defining and Addressing Current Safety Concerns", *Clinical Infectious Diseases,* (2001), 33(Suppl 4):S312-8; copy enclosed), states: "Historical problems with vaccines, including intussus-ception after rotavirus vaccine, carrier supression with tetanus toxoid conjugate vaccines, and decreased immunogenicity of some *Haemophilus influenzae* type b conjugate vaccines when mixed with acellular pertussis-diphtheria-tetanus, have contributed to some misperceptions about current vaccines. There is no evidence that adding additional vaccines through combination products increases the burden on the immune system, which has the capability of responding to many millions of antigens" (see Abstract).

In addition, applicants bring to the attention of the Examiner, In re Anderson, 176 USPQ 3331 (CCPA 1973). In this case, claims to a laminated dressing wherein the primary layer contains a medicament were rejected under 35 U.S.C. 112, first paragraph, as broader than the enabling disclosure because the term "medicament" was not limited to operative or suitable embodiments. However, the court reversed the rejection, holding that common sense would lead one of ordinary skill in the art to use operable embodiments:

> The concept of medicament or medication involves a highly technical subject in an art requiring a high degree of technical skill – doctors of medicine and pharmacologists. It is common knowledge

> wrong quantity, that one man's medicine is another man's poison, and that what is good medicine in one place may be bad medicine in another. The board, seemingly, is demanding a claim limitation to operative medicaments in operative quantity. We think that dependent claims such as the above, which merely add a limitation to the two-layer combination dressing by calling for medication in the primary layer, are inherently limited – by common sense if nothing else –to such medication as would be useful in the particular application. No one of ordinary skill in the art would use any other kind of medicament and there is no practical way to restrict the claim language so as to exclude all inoperative or deletrious medicaments other than by the addition of such redundant terms as "suitable" or "operative for the purposes described."...We are here dealing with combination claims, not with claims for medicaments per se. It is always possible to put something into a combination to render it inoperative. It is not the function of claims to exclude all such matters but to point out what the combination is. (471 F.2d 1237; 176 USPQ (BNA) 331; CCPA (1973)).

Claims 25 and 40 are combination claims of either a pharmaceutical or vaccine composition where the claims add a further element by reciting that the compositions further comprise additional components selected from the group consisting of DTP, DTaP, Td, DTaP-Hib, and DTaP-IPV-Hib. Although the publications cited by the Examiner report some concerns regarding combination vaccines, other combination vaccines are efficacious and widely used, and the Examiner has failed to provide any specific evidence challenging the utility of applicants' claimed invention.

In sum, the clinical significance of epitope suppression is still unclear in the art. However, the specification does provide guidance as to how to determine whether vaccines can elicit the production of antibodies that would be protective, i.e., by opsonophagocytic assays (page 23). These assays will determine whether antibodies elicited by a vaccination, including vaccinations with combined vaccines, are bactericidal and thus protective. Thus, applicants assert that claims 25 and 40 are dependent claims that specify further elements for N-propionated polysaccharide/oligosacharide-protein conjugate combined vaccine and pharmaceutical compositions; and that the specification enables one skilled in the art to determine whether such compositions may elicit bactericidal antibodies and thus warrant further "operative" clinical testing.

Response to Section 112, First Paragraph Rejection (Examiner's Action #10):

Claims 37-40 have been rejected under 35 U.S.C. §112, first paragraph, because the Examiner contends that the specification does not reasonably provide enablement for conjugate vaccines comprising N-acryloylated polysaccharide or oligosaccharide that provide protective immunity against any disease causing organism or cell. Applicants have amended claim 37 such that claims 37-40 now pertain to conjugate vaccines comprising N-acryloylated polysaccharides or oligosaccharides that provide protective immunity against at least one member of the genus of the organism from which the polysaccharide or oligosaccharide component of the polysaccharide-protein conjugate or oligosaccharide-protein conjugate was obtained. Applicants respectfully request reconsideration and removal of this rejection.

Response to Section 112, Second Paragraph Rejection (Examiner's Action #11(a-i)):

(a) Claims 1-5, 8-28 and 38-40 have been rejected under 35 U.S.C. §112, second paragraph, as being indefinite, for failing to particularly point out and distinctly claim subject matter.

Specifically, claims 1 and 16 have been rejected because the Examiner considers the recitation "directly conjugated to" or "directly conjugated" as unclear as to what direct conjugation encompasses.

The recitations "directly conjugated to" or "directly conjugated" refer to conjugations where no spacer or any such molecule is used to couple the polysaccharide or oligosaccharide to the protein. To clarify the application in this regard, applicants have amended the claims so that recitations of "direct conjugation" or "directly conjugated", now state "direct coupling" or "directly coupled". Applicants respectfully request withdrawal of this ground of rejection.

(b) Claims 2-5, 8 and 11-13 have been rejected for lacking proper antecedence for the recitation "A polysaccharide-protein conjugate according to claim...". Applicants have followed the Examiner's suggestion, and have amended the claims to recite –The polysaccharide-protein conjugate according to claim...--. Applicants respectfully request withdrawal of this ground of rejection.

(c) Claims 38-40 have been rejected for lacking proper antecedence for the recitation "A vaccine according to claim...". Applicants have amended the recitation according to the Examiner's suggestion: --The vaccine according to claim...--. Applicants respectfully request withdrawal of this ground of rejection.

(d) Claims 3-5 and 17 have been rejected because the Examiner contends that the term "derived" is vague and indefinite. Applicants have amended the claims by adding the term "obtained" in order to clarify the meaning of "derived". As stated earlier, "obtained" encompasses, for example, extraction, separation and purification; and "derived" encompasses, for example, modification. Applicants respectfully request withdrawal of this ground of rejection.

(e) Claim 20 has been rejected because the Examiner states that the recitation "carbonate/bicarbonate buffer" is unclear as to what this limitation encompasses. Applicants have amended claim 20 so that the recitation now states "bicarbonate buffer". Applicants respectfully request withdrawal of this ground of objection.

(f) Claim 24 has been rejected because the claim contains incorrect Markush language. Claim 24 has been amended to address the Examiner's concern. Applicants respectfully request withdrawal of this ground of objection.

(g) Claims 25 and 40 have been rejected because these claims have abbreviations that were unspecified in their full terminology. Claim 25 has been amended so that full terminology is used with the abbreviations retained in parenthesis. Applicants respectfully request withdrawal of this ground of objection.

(h) Claims 5 and 15 have been rejected for the inconsistent recitations of "type III" and "serotype III". Applicants have amended claim 5 to recite "type III" in order to maintain consistency with claim 15. Applicants respectfully request withdrawal of this ground of objection.

(i) Claims 9, 10, 14 and 18-28 have been rejected because of the vagueness of the base claim, claim 1. Applicants have amended claim 1 to address the Examiner's concerns. Applicants respectfully request withdrawal of this ground of objection.

Response to Section 102(b) Rejection, (Examiner's Action #13):

Claims 1-4, 8, 11-14, 22 and 26-28 have been rejected under 35 U.S.C. §102(b) as being anticipated by Roy *et al.* (*J. Chem. Soc. Chem. Commun.* 264-265, 1993), "Roy *et al.*, (1993)". The Examiner contends that Roy *et al.* (1993) reports a polysaccharide conjugate vaccine comprising an N-acryloylated mono- and poly-alpha-(2,8)-sialic acid or colominic acid antigen directly conjugated to a protein. Although applicants respectfully disagree with this rejection, applicants have amended the claims in order to more distinctly claim the instant invention.

All of the elements of amended claim 1 do not read upon the reference cited by the Examiner. Claim 1 encompasses a polysaccharide-protein conjugate or oligosaccharide-protein conjugate comprising an N-propionated saccharide (polysaccharide or oligosaccharide) directly conjugated (i.e., coupled) to a protein at the β-position of a propionate moiety. Roy *et al.* (1993) differs from the instant invention because Roy *et al.* reports conjugations that occur through the reducing end of carbohydrates: "The conjugations of the poly-α-(2,8)-sialic acid **4** to poly-L-lysine **3** and to the protein carriers were accomplished through its reducing end after derivatization to an *N*-acrylamide functionality." (p. 264, emphasis added). In contrast, the claimed invention describes direct coupling not at a reducing end after derivatization, but coupling at the β-position of a propionate moiety.

In the claimed invention, the propionate moiety is formed on one or more saccharides (i.e., oligosaccharides or polysaccharides) by de-acetylation followed by N-acryloylation at the same terminus that was de-acetylated (or "re-N-acryloylation"). Since an oligosaccharide or polysaccharide contains an acetyl group for every sugar residue, the instant invention allows for saccharides to be coupled to multiple protein molecules, or for saccharides to couple to a single protein molecule through multiple sites. The instant specification reveals this novel attribute of the invention, as it states: "The resulting N-acryloylated polysaccharide or N-acryloylated oligosaccharide is at least about 95% acryloylated or greater" (p. 9, lines 6-7),

which allows for the statements, "This invention provides the ability to produce conjugate molecules wherein the protein is linked to the polysaccharide or oligosaccharide through one or more sites on the polysaccharide or oligosaccharide...One or a multiplicity of polysaccharides or oligosacharides may cross-link with one or a multiplicity of protein" (page 9, line 30, to page 10, line 2). In contrast, the conjugates of Roy *et al.* (1993) do not allow multiple couplings because the N-acryloylation occurs at the reducing end of the saccharide.

To further clarify the coupling reaction that may occur for the claimed conjugates, applicants have duplicated Figure 2.11 from the reference: Pon, R.A. (*The Study of Polysialic acid Conjugates.* Master's Thesis, University of Ottawa, pp. 1-251, UMI Dissertation Services, 1992):

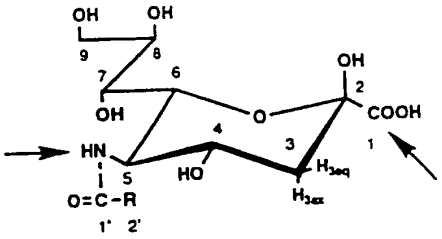


Figure 2.11- Derivatization points of sialic acid.

Depicted above is the repeating unit of colominic acid, where the large arrows represent the termini at which saccharides may be coupled, i.e. "derivatization points". Attached to carbon 5 is an acetyl group. In the instant invention, this acetyl group is de-N-acetylated and re-N-acryloylated, resulting in the formation of a propionate group at carbon 5 (in other repeating sugar residues, the terminus of de-N-acetylation/re-N-acryloylation may occur at a different carbon). This propionate group then serves as the nucleophilic acceptor in the Michael addition-mediated coupling reaction such that a protein is directly coupled to the saccharide at this terminus. Other reported methods of conjugation utilize Michael addition mediated coupling at the reducing terminus, in this example, carbon 1. Therefore, applicants respectfully request reconsideration and removal of this ground of rejection.

Response to Section 102(b) Rejection, (Examiner's Action #14):

Claims 1-3, 14 and 22 have been rejected under 35 U.S.C. §102(b) as being anticipated by Roy *et al.* (*J. Chem. Soc. Chem. Commun.* 536-538, 1991), "Roy *et al.* (1991)". The Examiner contends that Roy *et al.* (1991) reports antigenic carbohydrate protein conjugates comprising synthesized N-acryloylated sugars directly conjugated at the beta position to a lysine-containing protein. Although applicants respectfully disagree with this rejection, applicants have amended the claims in order to more distinctly claim the instant invention.

Roy *et al.* (1991) does not disclose polysaccharide or oligosaccharide-protein conjugates wherein the coupling between the saccharide and protein is direct. Scheme 1, on page 537 of Roy *et al.* (1991), show coupling of a sugar to a protein via a spacer molecule, namely a phenyl group. In contrast, the instant invention claims conjugates wherein the coupling between a saccharide and a protein is direct, without the use of a chemical spacer.

Secondly, Roy *et al.* (1991) does not disclose conjugates wherein the coupling can occur at non-reducing ends of saccharides. Rather, Roy *et al.* (1991) shows that N-acryloylation occurs at the amino group of the phenyl-spacer, and subsequent coupling of the protein occurs at this reducing end. In contrast, the instant invention claim conjugates wherein the coupling occurs at non-reducing ends, namely at de-N-acetylated/re-N-acryloylated termini. Applicants respectfully request reconsideration and withdrawal of this ground of rejection.

Response to Section 102(b) Rejection, (Examiner's Action #15):

Claims 1-4, 8, 11-14, 16, 17 and 19-22 have been rejected under 35 U.S.C. §102(b) as being anticipated by Pon, R.A. (*The Study of Polysialic acid Conjugates.* Master's Thesis, University of Ottawa, pp. 1-251, UMI Dissertation Services, 1992). The Examiner contends that Pon reports polysaccharide or oligosaccharide-protein conjugates produced by a method comprising de-N-acetylating saccharides using a de-N-acetylating base reagent, followed by N-acryloylating the de-N-acetylated saccharide with an acryloylating reagent, and directly conjugating the resultant saccharide to a protein. Although applicants respectfully disagree with this rejection, applicants have amended the claims in order to more distinctly claim the instant invention.

Pon reports the conjugation of N-acryloyl colominic acid onto BSA or IgG, wherein the colominic acid is 15% N-acryloylated. For example, at page 181, Pon states: "BSA

(4-17) (5 mg) or IgG (4-36) (5 mg) was combined with 15% N-acryloylated colominic acid (4-16) (10 mg) and dissolved in 200 µl borate buffer (0.1M; pH 8.3)." However, Pon does not report that this conjugate can stimulate a productive response. Because the sugar is only 15% acryloylated, the degree of protein coupling is limited and will therefore negatively impact the effectiveness of an immune response.

In contrast, the conjugates of the instant application are highly acryloylated and have been shown to produce productive immune responses. For instance, the specification states the degree of acryloylation of the claimed N-propionated saccharides: "The resulting N-acryloylated polysaccharide or N-acryloylated oligosaccharide is at least about 95% acryloylated or greater." (page 9, first paragraph). Also, the specification discloses the immunogenicity of β-propionamido-linked polysaccharide-protein conjugates in Tables 5-8 (pages 24-27), as productive immune responses are observed by ELISA and opsonophagocytic assays. Therefore, applicants have amended the claims by adding the recitation "elicits protective antibodies", in order to more explicity show the advantages and novelty of the instant invention. Applicants respectfully request reconsideration and withdrawal of this ground of rejection.

Response to Section 102(b) Rejection, (Examiner's Action #16):

Claims 1-3, 8, 11-14 and 22 have been rejected under 35 U.S.C. §102(b) as being anticipated by Roy *et al.* (*J. Chem. Soc. Chem. Commun.* 1709-1711, 1990), "Roy *et al.* (1990)". The Examiner contends claims 1-3, 8, 11-14 and 22 have been anticipated because Roy *et al.* (1990) reports conjugates comprising N-acryloylated sialic acid- and sialyloligosaccharide-protein lactoside directly conjugated to proteins by Michael addition. Although applicants respectfully disagree with this ground of rejection, applicants have amended the claims in order to more distinctly claim the novelty of the present invention.

Claims 1-3, 8, 11-14 and 22 are not anticipated by Roy *et al.* (1990) because the present invention relates to polysaccharide- and oligosaccharide-protein conjugates, wherein the formation of these conjugates is dependent upon direct coupling of saccharides and proteins by Michael addition at non-reducing termini. The coupling at the non-reducing termini is contingent upon the de-acetylation and re-N-acryloylation at the same terminus that was de-acetylated. The de-acetylation and re-N-acryloylation results in a propionate group at the terminus that originally had a acetyl group, and the propionate moiety acts as a nucleophilic

acceptor in the Michael addition reaction that couples the saccharide to a protein. In contrast, Roy *et al.* (1990) reports coupling between saccharides and proteins where the coupling occurs at the reducing termini of saccharides (see Scheme 3). Applicants respectfully request reconsideration and withdrawal of this ground of rejection.

Response to Section 102(b) Rejection, (Examiner's Action #17):

Claims 1-3, 8, 11-14 and 22 have been rejected under 35 U.S.C. §102(b) as being anticipated by Romanowska *et al.* (*Methods in Enzymol.* 242:90-101, 1994). The Examiner contends that Romanowska *et al.* reports artificial N-acryloylated sialic acid, sialoside and a T antigen derivative directly conjugated to BSA, tetanus toxoid or poly(L-lysine) via epsilon amino groups.

Although Romanowska reports conjugation of proteins onto N-acryloylamido substituted glycosides, Romanowska does not disclose that such conjugations may occur on non-reducing termini. The instant invention claims conjugations wherein the coupling of protein and saccharide occurs at propionamido termini, where these termini have been de-N-acetylated and re-N-acryloylated. Therefore, the claims have been amended to more distinctly claim the instant invention by making clear that the coupling does not occur at reducing termini. Applicants respectfully request reconsideration and withdrawal of this ground of rejection.

Response to Section 102(b) Rejection, (Examiner's Action #18):

Claims 1-4, 11-14 and 22 have been rejected under 35 U.S.C. §102(b) as being anticipated by Auzanneau *et al.* (*Bioorg. Medicinal Chem.* 4:2003-2010, 1996). The Examiner has rejected these claims because Auzannneau *et al.* reports N-acryloylated Group A streptococcal cell wall oligosaccharide conjugated to BSA or OVA by the addition of ε-amino groups of lysines present on protein. Applicants respectfully disagree with this ground of rejection.

Applicants have amended the claims to clarify the novelty of the instant invention. The claimed conjugates are formed by a distinct process: de-acetylation, N-acryloylation at the termini that are de-acetylated (thereby forming a propionate moiety at these termini), and direct coupling of proteins, via Michael addition, to the β–position of the propionate moiety(ies). In contrast, Auzanneau *et al.* reports conjugation wherein coupling of

protein and saccharide occurs at N-acryloylated termini that have not been de-acetylated (see Figures on 2004). Applicants respectfully request reconsideration and withdrawal of this ground of rejection.

Response to Section 102(b) Rejection, (Examiner's Action #19):

Claims 1-3, 8 and 11-14 have been rejected under 35 U.S.C. §102(b) as being anticipated by Roy *et al.* (*Bioorg. Medicinal chem. Lett.* 2:911-914, 1992), "Roy *et al.* (1992)". The Examiner contends that claims 1-3, 8 and 11-14 are anticipated because Roy *et al.* (1992) reports neoglycoproteins comprising N-acryloylated carbohydrate T antigen, or a blood group trisaccharide determinant conjugated by Michael addition to a protein carrier.

Applicants respectfully disagree with this ground of rejection, as applicants' amendments to the claims have clarified that the present invention pertains to conjugates that are formed by the direct coupling of proteins to propionate groups at non-reducing termini of saccharides, where these propionate groups have been formed by de-acetylation and re-N-acryloylation. In contrast, Roy *et al.* (1992) report indirect coupling, i.e. via a spacer, of proteins to the reducing end of saccharides. Therefore applicants respectfully request reconsideration and withdrawal of this ground of rejection.

Response to Section 103(a) Rejections, (Examiner's Actions #21 and #22):

Claims 1 and 8-10 have been rejected under 35 U.S.C. §103(a) as being unpatentable over Pon, R.A. (*The Study of Polysialic acid Conjugates.* Master's Thesis, University of Ottawa, pp. 1-251, UMI Dissertation Services, 1992) in view of Blake *et al.* (U.S. Patent No. 5,439,808). The Examiner contends that the combination of Pon and Blake *et al.* makes obvious saccharide-protein conjugates, wherein the protein is a *N. meningitidis* outer membrane protein.

Claims 1, 16 and 22-24 have been rejected under 35 U.S.C. §103(a) as being unpatentable over Pon, R.A. (*The Study of Polysialic acid Conjugates.* Master's Thesis, University of Ottawa, pp. 1-251, UMI Dissertation Services, 1992) in view of Blake *et al.* (U.S. Patent No. 5,439,808). The Examiner contends that the combination of Pon and Blake *et al.* makes obvious pharmaceutical compositions comprising saccharide-protein conjugates and adjuvants.

Applicants respectfully disagree with these grounds of rejection because Pon does not teach or suggest all the claim limitations. As stated previously, Pon reports the conjugation of N-acryloyl colominic acid to BSA or IgG, wherein the colominic acid is 15% N-acryloylated. However, Pon does not report that this conjugate can stimulate a productive response. Because the sugar is only 15% acryloylated, the degree of protein coupling is limited and will therefore negatively impact the effectiveness of an immune response.

In contrast, the conjugates of the instant application are highly acryloylated and have been shown to produce productive immune responses. For instance, the specification states the degree of acryloylation of the claimed N-propionated saccharides: "The resulting N-acryloylated polysaccharide or N-acryloylated oligosaccharide is at least about 95% acryloylated or greater." (page 9, first paragraph). Also, the specification discloses the immunogenicity of β-propionamido-linked polysaccharide-protein conjugates in Tables 5-8 (pages 24-27), as productive immune responses are observed by ELISA and opsonophagocytic assays. Therefore, applicants have amended the claims by adding the recitation "elicts protective antibodies", in order to more explicity show the advantages and novelty of the instant invention.

Thus, the combination of Pon in view of Blake *et al.* does not teach or suggest all of the limitations of the claimed invention. Applicants respectfully request reconsideration and withdrawal of this ground of rejection.

Response to Objections, (Examiner's Actions #23(a)-(g)):

(a) Claims 37 and 38 have been objected to due the recitation "disease causing organism". This recitation has been deleted from the claims.

(b) Claims 5 and 8 have been objected to for inconsistent recitation of "Group B streptococcus" and "group B *Streptococcus*". Claims 5 and 8 have been amended to recite "group B *Streptococcus*".

(c) Claim 15 has been objected to for inconsistent recitations as in (b). Claim 15 has been amended as in (b).

(d) Claim 26 has been objected to for the incorrect recitation "A immunogen". Claim 26 has been amended to recite "An immunogen".

(e) Claims 22 and 26 have been objected to with regard to the recitation "claims 1 or 16". Claims 22 and 26 have been amended to recite "any one of claim 1 or claim 16".

16". Claims 22 and 26 have been amended to recite "any one of claim 1 or claim 16".

(f) Claim 5 has been objected to for being dependent from a rejected claim (claim 1). Claim 1 has been amended.

(g) Claim 15 has been objected to for including one or more non-elected inventions. Claim 15 is further objected to for lacking antecedent basis in the specification for the limitation: "N-propionated". Claim 15 has been amended to eliminate inclusion of non-elected inventions. In reference to the objection of "N-propionated", please refer to applicants' response to Examiner's action #7(c)). Applicants respectfully request reconsideration and withdrawal of the above grounds of objection.

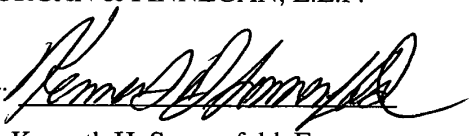
AUTHORIZATION

No additional fee is believed to be necessary. The Commissioner is hereby authorized to charge any additional fees which may be required for this amendment, or credit any overpayment to Deposit Account No. 13-4500, Order No. 3842-4043US1. A DUPLICATE COPY OF THIS PAGE IS ATTACHED.

Respectfully submitted,

MORGAN & FINNEGAN, L.L.P.

Dated: April 23, 2002

By: 

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APPENDIX:

VERSION OF THE AMENDMENTS TO THE CLAIMS AND SPECIFICATION SHOWING DELETIONS AND ADDITIONS

IN THE CLAIMS:

Claims 1-5, 8, 11-22, 24-26, and 37-40 have been amended as follows:

1. A polysaccharide-protein conjugate or oligosaccharide-protein conjugate that elicits protective antibodies wherein said conjugates [comprising] comprise [an N-propionated polysaccharide or N-propionated oligosaccharide] an N-propionated saccharide directly [conjugated] coupled to a protein at [the] a β-position of [the] a propionate moiety, and wherein the N-propionated saccharide is de-N-acetylated and N-acryloylated at the de-N-acetylated terminus.

2. [A polysaccharide-protein conjugate] The conjugates according to claim 1 wherein the protein comprises at least one lysine or cysteine residue.

3. [A polysaccharide-protein conjugate or oligosaccharide-protein conjugate] The conjugates according to claim 1 wherein the [polysaccharide or oligosaccharide] saccharide is derived from a polysaccharide obtained from bacteria, yeast, cancer cells, or is chemically synthesized.

4. [A polysaccharide-protein conjugate or oligosaccharide-protein conjugate] The conjugates according to claim 1 wherein the [polysaccharide or oligosaccharide] saccharide is derived from a polysaccharide obtained from *Escherichia coli*, Meningococcus, Pneumococcus, Streptococcus, Haemophilus, Neisseria, Salmonella, Klebsiella, or Pseudomonas.

5. [A polysaccharide-protein conjugate or oligosaccharide-protein conjugate] The conjugates according to claim 1 wherein the [polysaccharide or oligosaccharide] saccharide is derived from a polysaccharide obtained [G]group B [streptococcus] *Streptococcus* selected from

the group consisting of [sero]type Ia, [sero]type Ib, [sero]type II, [sero]type III, [sero]type V, [sero]type VIII, and combinations thereof.

8. [A polysaccharide-protein conjugate or oligosaccharide-protein conjugate] The conjugates according to claim 1 wherein the protein is selected from the group consisting of tetanus toxoid, diphtheria toxoid, a *Neisseria meningitidis* outer membrane protein, pneumolysoid, C-β protein from group B *Streptococcus* and non-IgA-binding C-ß protein from group B *Streptococcus*.

9. [The polysaccharide-protein conjugate or oligosaccharide-protein conjugate] The conjugates according to claim 8 wherein the protein is recombinantly produced.

10. [The polysaccharide-protein conjugate or oligosaccharide-protein conjugate] The conjugates according to claim 9 wherein the protein is recombinant *N. meningitidis* outer membrane protein.

11. [A polysaccharide-protein conjugate or oligosaccharide-protein conjugate] The conjugates according to claim 1 wherein the [polysaccharide or oligosaccharide] saccharide comprises a glycosaminoglycan.

12. [A polysaccharide-protein conjugate or oligosaccharide-protein conjugate] The conjugates according to claim 1 wherein the [polysaccharide or oligosaccharide] saccharide comprises glycosyl residues of a structural repeating unit having at least one free amino group or N-acyl group.

13. [A polysaccharide-protein conjugate or oligosaccharide-protein conjugate] The conjugates according to claim 12 wherein the glycosyl residue is selected from the group consisting of glucosamine, galactosamine, mannosamine, fucosamine and sialic acid.

14. The [polysaccharide-protein conjugate or oligosaccharide-protein conjugate] conjugates according to claim 1 wherein the N-propionated [polysaccharide or N-propionated

oligosaccharide] saccharide is directly [conjugated] coupled to an ε-free amino group of a lysine residue or a thiol group of a cysteine residue of the protein.

15. A polysaccharide-protein conjugate or oligosaccharide-protein conjugate comprising [N-propionated *Streptococcus pneumoniae* type 14 polysaccharide-tetanus toxoid conjugate,] N-propionated [G]group B [streptococcus] a *Streptococcus* type III polysaccharide-tetanus toxoid conjugate[, N-propionated Group B streptococcus type II polysaccharide-tetanus toxoid conjugate, N-propionated *E. coli* K1 polysaccharide-protein conjugate, or N-propionated meningococcal C polysaccharide-tetanus toxoid conjugate].

16. A polysaccharide-protein conjugate or oligosaccharide-protein conjugate that elicits protective antibodies produced by a method comprising:

A) de-N-acetylating an isolated polysaccharide or oligosaccharide using a de-N-acetylating reagent to form a de-N-acetylated polysaccharide or a de-N-acetylated oligosaccharide,

B) N-acryloylating the de-N-acetylated polysaccharide or the de-N-acetylated oligosaccharide at a de-N-acetylated terminus with an acryloylating reagent to form an N-propionated polysaccharide or an N-propionated oligosaccharide, and

C) directly [conjugating] coupling at a β-position of a propionate moiety of the N-propionated polysaccharide or [an] the N-propionated oligosaccharide to a protein to form the polysaccharide-protein conjugate or the oligosaccharide protein conjugate.

17. The [polysaccharide-protein conjugate or oligosaccharide-protein conjugate] conjugates according to claim 16 wherein the polysaccharide or oligosaccharide is obtained [derived] from bacteria, yeast, or cancer cells or is [chemical synthesis] chemically synthesized.

18. The [polysaccharide-protein conjugate or oligosaccharide-protein conjugate] conjugates of claim 16 wherein the [conjugation] coupling is conducted at a pH of about 7.0.

19. The [polysaccharide-protein conjugate or oligosaccharide-protein conjugate] conjugates of claim 16 wherein the [conjugation] coupling is conducted at a pH above 9.

20. The [polysaccharide-protein conjugate or oligosaccharide-protein conjugate] conjugates of claim 16 wherein the [conjugation] coupling is conducted in a reagent selected from the group consisting of phosphate buffer, [carbonate/]bicarbonate buffer, and borate buffer.

21. The [polysaccharide-protein conjugate or oligosaccharide-protein conjugate of] conjugates according to claim 16 wherein the de-N-acetylating reagent is a base or an enzyme and the acryloylating reagent is selected from the group consisting of N-acryloyl chloride, acryloyl anhydride, acrylic acid and a dehydrating agent.

22. A pharmaceutical composition comprising the [polysaccharide-protein conjugate or oligosaccharide-protein conjugate] conjugates according to any one of claim[s] 1 or claim 16 and a pharmaceutically acceptable carrier.

24. The pharmaceutical composition according to claim 23 wherein the adjuvant is selected from the group consisting of alum [or] and stearyl tyrosine.

25. The pharmaceutical composition according to claim 22 further comprising a second component, said second component selected from the group consisting of diphtheria-tetanus-pertussis (DTP), diphtheria-tetanus-acellular pertussis (DTaP), tetanus-diphtheria (Td), diphtheria-tetanus-acellular pertussis-*Haemophilus influenzae type B* (DTaP-Hib), diphtheria-tetanus-acellular pertussis-inactivated poliovirus-*Haemophilus influenzae type B* (DTaP-IPV-Hib), and combinations thereof.

26. [A] An immunogen comprising the [polysaccharide-protein conjugate or oligosaccharide-protein conjugate] conjugates according to any one of claim[s] 1 or claim 16, said immunogen elicits a polysaccharide-specific or an oligosaccharide-specific immune response.

37. A vaccine comprising the [polysaccharide-protein conjugate or oligosaccharide-protein conjugate] conjugates according to any one of claim 1 or claim 16, wherein said vaccine

provides protective immunity against [a disease causing organism or cell] at least one member of a genus of an organism from which the polysaccharide or oligosaccharide component of the polysaccharide-protein conjugate or oligosaccharide-protein conjugate was extracted.

38. [A] The vaccine according to claim 37 wherein the [disease causing] organism [or cell] is selected from the group consisting of bacteria[,] and yeast[, and cancer cell].

39. [A] The vaccine according to claim 38 wherein the bacteria is selected from the group consisting of *Escherichia coli*, Meningococcus, Pneumococcus, Streptococcus, Haemophilus, Neisseria, Salmonella, Klebsiella, [or] and Pseudomonas.

40. [A] The vaccine according to claim 37 further comprising a second immunogen in combination with the polysacharide-protein conjugate or oligosaccharide-protein conjugate said second immunogen selected from the group consisting of DTP, DTaP, Td, DTaP, Hib, DTaP-IPV-Hib and combinations thereof.

IN THE SPECIFICATION:

On page 17, line 24 has been rewritten as follows:
-- the miniDAWN® (Wyatt Technology Corp., Santa Barbara, CA) --

On page 18, line 22 has been rewritten as follows:
-- 1.6x60cm column of [Superdex] SUPERDEX™ 200 PG (Pharmacia) and eluted with PBS containing --

On page 19, line 19 has been rewritten as follows:
-- deionized water. After diafiltration through an [Amicon] AMICON™ YM3 membrane with deionized --

On page 20, line 6 has been rewritten as follows:

-- N HCL. The solution was diafiltrated with an [Amicon] AMICON™ YM3 membrane in a stircell --

On page 20, line 13 and line 14 have been rewritten as follows:
-- [Zwittergen™] ZWITTERGEN™ 3,14 (Boehringer Mannheim) pH 9.5 was incubated at 37°C for 3 days. The conjugate was purified by size exclusion chromatography through a [Superdex] SUPERDEX™ 200 --

On page 20, line 30 has been rewritten as follows:
-- chloride and 0.05% [Zwittergent] ZWITTERGEN™ 3-14 and loaded onto a [Pharmacia] PHARMACIA™ PD-10 desalting --

On page 21, line 1 has been rewritten as follows:
-- [Amicon] AMICON™ Centricon® 30 concentrator at 5,000 RPM for one hour. The retentate was --

On page 21, line 9 has been rewritten as follows:
-- [Superdex] SUPERDEX™ 200 column (Pharmacia) with PBS as eluant. UV-280-nm-active --

On page 22, lines 28 and 29 have been rewritten as follows:
-- hour at 37°C, followed by a PBS-[Tween] TWEEN™ (0.05% v/v [Tween] TWEEN™ 20 in PBS) was (5 times). All subsequent incubations were conducted at room temperature. PBS-[Tween] TWEEN™ --

On page 23, line 9 has been rewritten as follows:
-- well, and the plate was read on a [Molecular Devices] MOLECULAR DEVICES™ Emax® microplate reader –

On page 9, line 1 has been rewritten as follows:
-- acryloyl anhydride, acrylic acid and a dehydrating agent such as

dicyclohexylcarbodiimide (DCC), $CH_2CHCOCN$ --

16". Claims 22 and 26 have been amended to recite "any one of claim 1 or claim 16".

(f) Claim 5 has been objected to for being dependent from a rejected claim (claim 1). Claim 1 has been amended.

(g) Claim 15 has been objected to for including one or more non-elected inventions. Claim 15 is further objected to for lacking antecedent basis in the specification for the limitation: "N-propionated". Claim 15 has been amended to eliminate inclusion of non-elected inventions. In reference to the objection of "N-propionated", please refer to applicants' response to Examiner's action #7(c)). Applicants respectfully request reconsideration and withdrawal of the above grounds of objection.

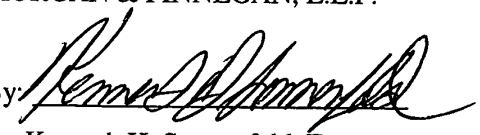
AUTHORIZATION

No additional fee is believed to be necessary. The Commissioner is hereby authorized to charge any additional fees which may be required for this amendment, or credit any overpayment to Deposit Account No. 13-4500, Order No. 3842-4043US1. A DUPLICATE COPY OF THIS PAGE IS ATTACHED.

Respectfully submitted,

MORGAN & FINNEGAN, L.L.P.

Dated: April 23, 2002

By: 

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CONCISE COMMUNICATIONS

Avidity Maturation of Antibody to *Haemophilus influenzae* Type b (Hib) after Immunization with Diphtheria-Tetanus–Acellular Pertussis–Hib–Hepatitis B Combined Vaccine in Infants

Michael E. Pichichero, Timothy Voloshen, Diane Zajac, and Sherry Passador

University of Rochester, Department of Microbiology/Immunology, Rochester, New York

Antibody avidity to *Haemophilus influenzae* type b (Hib) polysaccharide (PS) was assessed in infants vaccinated with diphtheria-tetanus–acellular pertussis (DTaP) combined with Hib-PS conjugated to tetanus toxoid (PRP-T) and hepatitis B (HB) (DTaP–PRP-T–HB) and after a PRP-conjugate (CRM_{197}-OS) booster 3–7 months later. Avidity differed between infants with anti–Hib-PS IgG antibody <1 or >1 μg/mL postprimary series (avidity index [AI], 42%, 95% confidence interval [CI], 35%–49%, and 68% and 63%–72%, respectively; $P < .0001$). For infants with <1 μg/mL anti–Hib-PS IgG antibody, mean AI rose by the time of preboost immunization to 61% (95% CI, 57%–65%), even though total IgG antibody levels fell. Spontaneous Hib-PS antibody rises after primary series DTaP–PRP-T–HB vaccination were followed by high post-boost anti–Hib-PS IgG antibody levels and avidity. The DTaP–PRP-T–HB combination vaccine studied elicits high avidity antibody, and affinity maturation appears to occur in the absence of further antigen exposure even in those with very low anti–Hib-PS antibody.

Haemophilus influenzae type b (Hib) conjugate vaccines prime infants for memory antibody responses to subsequent Hib polysaccharide (PS) encounter [1–4]. Diphtheria-tetanus–acellular pertussis vaccines (DTaP) combined with Hib conjugate vaccines (with or without hepatitis B; HB) administered to 2-, 4-, and 6-month-old infants produce lower Hib-PS antibody levels than simultaneous but separate injections [1, 5, 6]. The biologic significance of diminished anti–Hib-PS antibody levels is debated [7, 8].

We previously examined anti–Hib-PS antibody responses after DTaP-Hib conjugated to tetanus toxoid (PRP-T)–HB combination vaccination in infants and characterized antibody responses to PRP-conjugate booster [1]. Even in those with low (<1.0 μg/mL) or undetectable (<0.10 μg/mL) postprimary anti–Hib-PS antibody levels, we found evidence for immunologic memory on the basis of high-titered, IgG-predominant responses after a PRP-conjugate booster (CRM_{197}-OS). Here we studied the anti–Hib-PS antibodies produced in the children after the CRM_{197}-OS booster for avidity and bactericidal function as additional measures of memory. We also compared avidity of anti–Hib-PS IgG antibody in child cohorts with higher and lower quantitative responses after DTaP–PRP-T–HB combination and separately injected vaccines and monitored avidity changes over time in low anti–Hib-PS responders to assess the pace of affinity maturation.

Study Design

Primary and booster immunization. Healthy infants participated in a multicenter prospective, randomized trial evaluating DTaP, PRP-T, and HB as separate vaccines given simultaneously or as a single combined vaccine (SmithKline Beecham Biologicals, Rixensart, Belgium) at ages 2, 4, and 6 months; the trial design has been described elsewhere [1]. Of the 331 children enrolled, 121 (37%) had anti–Hib-PS antibody levels <1.0 μg/mL 1 month after the third vaccination (when vaccinees were 7 months old). Of these, 43 children in Rochester, New York, were given a Hib-PS conjugate booster (CRM_{197}-OS; Wyeth-Lederle Vaccines and Pediatrics, Rochester, NY) at ages 9–13 months (before and 1 month after PRP-conjugate booster sera were obtained). For comparison purposes, a random subset of 28 sera from Rochester infants with anti–Hib-PS >1.0 μg/mL were analyzed; 14 sera were from children who had received the combined vaccine, and 14 were from children who had received separate vaccines simultaneously at the designated visits.

Vaccines. All vaccines used in the trial were described elsewhere [1].

Assay methods. All assays were performed at the investigators' University of Rochester laboratory.

Hib ELISA. Anti–capsular (anti-polyribosylribitolphosphate; Hib-PS) IgG antibody was quantitated by ELISA as described by

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Presented in part: Academic Pediatric Societies meeting, New Orleans, 3 May 1998.

Written informed consent was obtained from parents or guardians for all trial participants.

Grant support: NIH (AI-45248).

Reprints or correspondence: Dr. Michael E. Pichichero, University of Rochester Medical Center, Elmwood Pediatric Group, 601 Elmwood Ave., Box 672, Rochester, NY 14642.

The Journal of Infectious Diseases 1999;180:1390–3

0022-1899/1999/18004-0066$02.00

Table 1. Comparison of IgG antibody concentration in infants at 3 time points.

Response to Hib-PS	Geometric mean anti-Hib-PS IgG antibody levels: After 3-dose primary series at age 2, 4, and 6 months with DTaP-PRP-T-HB combination vaccine at age 7 months	Before booster at age 9–13 months	1 month after booster with CRM_{197}-OS Hib conjugate
Low (n=43)[a]	0.54 (0.47–0.61)	0.33 (0.26–0.40)	7.94 (0.73–15.2)
High (n=28)[a]	6.8 (4.67–8.95)	ND[b]	ND[b]

NOTE. Data are μg/mL (95% confidence interval). Hib, *Haemophilus influenzae* type b, PS, polysaccharide; DTaP-PRP-T-HB, diphtheria-tetanus-acellular pertussis conjugated to tetanus toxoid and hepatitis B.

[a] Low responders to Hib-PS vaccine defined as occurring if <1.0 μg/mL anti-Hib-PS antibody 1 month after 3 doses of DTaP-PRP-T-HB combination vaccines at ages 2, 4, and 6 months; high responders had ≥1.0 μg/mL.

[b] ND, not done because booster not given.

Phipps et al. [9] using Hib oligosaccharide–human serum albumin conjugate (Wyeth-Lederle; lower limit of quantitation, 0.10 μg antibody/mL; assay coefficient of variation, 12%, 420 determinations). CBER standard lot 1983 containing 70 μg/mL total anti–Hib-PS antibody was the reference standard.

Avidity assay. Anti–Hib-PS IgG avidity was quantitated by ELISA by the method of Agbarakwe et al. [10] with modifications. Hib oligosaccharide–human serum albumin was diluted to 1 μg/mL in 0.05 *M* carbonate buffer and bound overnight at 37°C to round-bottom microtiter plates (Immulon II; Dynatech, Chantilly, VA). Duplicate serum samples were then serially diluted (2-fold) in PBS–0.05% Tween; plates were incubated 1 h at room temperature. After 5 PBS–0.1% Tween washes, 6.0 *M* urea was added to 1 row of sera and PBS–0.05% Tween to a corresponding row of the same sera. Calculation of anti–Hib-PS antibody was based on the reference line method compared with the CBER standard 60.9 μg/mL IgG (provided by Carl Frasch, US Food and Drug Administration, Washington, DC). The avidity index (AI; expressed as percentage) was calculated as anti–Hib-PS IgG antibody concentration after urea exposure divided by anti–Hib-PS IgG antibody concentration without urea exposure × 100. The avidity assay could not be done on sera with <0.3 μg/mL IgG anti–Hib-PS antibody. The CBER standard, a pool of normal, healthy adult sera, had a mean AI of 59% (confidence interval [CI], 57%–61%) with a 6% coefficient of variation (13 determinations).

Bactericidal assay. For the bactericidal assay, we used the method of Anderson et al. [11]. The bactericidal end-point titer was the highest serum dilution producing a 50% colony reduction versus control; results are expressed as the reciprocal dilution. Titers <8 were assigned a titer of 4.

Statistical analysis. Unpaired and paired Student's *t* tests were used, as appropriate, to compare anti–Hib-PS antibody levels and avidity between vaccination time points (after primary and before and after boosting). Arithmetic and log-transformed values were used as appropriate. Sample AIs were considered above the CBER standard if they were above the CBER 95% upper CI, the same as the CBER standard if they were within the CBER 95% CI, and lower if they were below the CBER 95% CI. Pearson's correlation coefficient was calculated for anti–Hib-PS IgG antibody and bactericidal activity (BA).

Results

Levels and antibody avidity after primary vaccination. After the primary series of DTaP-Hib-HB vaccinations, the geometric mean (GM) and 95% CI for anti–Hib-PS IgG antibody level for the infant cohort with <1.0 μg/mL anti–Hib-PS antibody (n = 43) was 0.54 μg/mL (CI, 0.47–0.61); for the cohort with >1.0 μg/mL (n = 28), it was 6.8 μg/mL (CI, 4.7–8.9; table 1). The AI in vaccinees with <1.0 μg/mL anti–Hib-PS IgG antibody was higher than the CBER standard after the primary series of DTaP-PRP-T-HB vaccinations in 5% of the infants, similar in 11%, and lower in 84%. In comparison, the AI in vaccinees with >1.0 μg/mL anti–Hib-PS IgG antibody was higher than the CBER standard in 71%, similar in 11%, and lower in 18%. Vaccinees with <1.0 μg/mL anti–Hib-PS antibody had a mean AI of 42% (CI, 35%–49%), which differed from the mean AI of 68% (CI, 63%–72%) for vaccinees with >1.0 μg/mL (P < .001; figure 1).

Preboosster antibody levels and avidity. GM anti–Hib-PS IgG antibody levels for the infant cohort with <1.0 μg/mL after primary vaccination was 0.33 μg/mL (CI, 0.26–0.40) before booster. While a significant decrease was observed in anti–Hib-PS antibody (P = .008), AI increased significantly in the time between postprimary (mean AI, 42%) and preboost immunization (mean AI, 62%, P < .001; figure 1). In fact, by the time of preboost sera sampling, there was no significant difference in mean AI when the infant cohort with <1.0 μg/mL anti–Hib-PS was compared with the cohort with >1.0 μg/mL anti–Hib-PS or to CBER reference (figure 1).

Postbooster antibody levels and avidity. All vaccinees showed an anti–Hib-PS antibody rise after the CRM_{197}-OS booster (GM, 7.94 μg/mL; CI, 0.73–15.2 μg/mL, although 2 children did not exceed 1.0 μg/mL after boosting). Avidity did not increase before boost (AI, 61%, n = 17, for those in whom it could be measured) to postboost levels (AI, 64%, n = 43, P = .5). However, when the subset of 17 children from whom we had paired pre- and postboost sera were analyzed, a rise in AI from 61% to 70% was observed (P = .01).

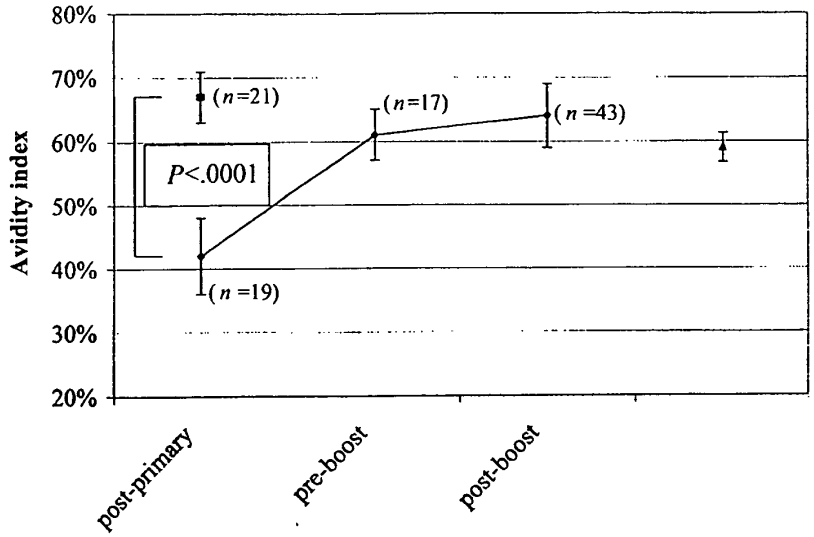

Figure 1. Mean avidity index (AI) and 95% confidence intervals are plotted for infant cohort with <1.0 μg/mL anti-*Haemophilus influenzae* type b (Hib)-polysaccharide (PS) IgG antibody (♦) after 3-dose series of diphtheria-tetanus-acellular pertussis conjugated to tetanus toxoid and hepatitis B (DTaP-PRP-T-HB) combination vaccine at ages 2, 4, and 6 months, before CRM_{197}-OS booster at age 9–13 months, and 1 month after booster. For comparison, mean AI for infant cohort with >1.0 μg/mL anti-Hib-PS IgG antibody after 3 doses of same DTaP-PRP-T-HB vaccine (■) and adult reference pool from CBER (▲) are shown.

From postprimary to prebooster immunization, 36 of 43 children had similar or diminished anti-Hib-PS antigen concentrations. The GM anti-Hib-PS antibody level and mean AI for these 36 children before boosting was 0.32 μg/mL and 58% (n = 11); after boosting, these were 7.09 μg/mL and 59%, respectively. Seven children with undetectable anti-Hib-PS levels after primary vaccination series showed rises in antibody before the CRM_{197}-OS booster. The GM anti-Hib-PS antibody level and mean AI for these 7 children before boosting was 0.36 μg/mL and 68% (n = 5), compared with 18.10 μg/mL and 78%, respectively, after boosting; these values were higher than for the 36 children who did not show such rises (P = .06 and .006 for GM and AI, respectively).

Bactericidal antibody determinations. BA against Hib was assessed in sera from infants with low postprimary anti-Hib-PS antibody levels. Mean BA increased significantly from 4 before boosting to 764 1 month after boosting (P = .002). Postbooster BA correlated with postboost anti-Hib-PS IgG antibody levels (r = .7, P < .001) but not with avidity of anti-Hib-PS IgG antibody (r = .10, P, not significant).

Discussion

There have been concerns about interference with Hib vaccine responses, manifested as a decrease in the anti-Hib-PS antibody level, when Hib and DTaP vaccines are combined [1, 5, 6]. We recently studied a DTaP-PRP-T-HB combination vaccine and found evidence that immunologic memory was induced even in infants with lower (<1.0 μg/mL) and even undetectable (<0.10 μg/mL) postprimary anti-Hib-PS antibody levels [1]. Thus, we suggested that the combination vaccine primed the infant immune system for anamnestic anti-Hib-PS antibody responses. Affinity maturation is another major feature of immunologic memory. Here, we showed that DTaP-PRP-T-HB vaccinees elicit high avidity IgG antibody against the Hib PS antigen. Unexpectedly, avidity increased most significantly in the 3–7 months after primary DTaP-PRP-T-HB vaccination, with a marginal further rise after a CRM_{197}-OS booster. In fact, before the Hib conjugate booster, the mean AI of low anti-Hib-PS responders had risen to a level similar to vaccinees with >1.0 μg/mL antibody after the primary vaccination series and to the CBER adult sera standard. Although there was some heterogeneity in avidity of anti-Hib-PS IgG antibody after combination vaccine in low anti-Hib-PS responders, just before boosting, all sera for which we could measure avidity would be considered to have relatively high-avidity anti-Hib-PS antibody [10]. Thus, it would appear that affinity maturation was slower in the lower anti-Hib-PS responders, but by the time of the booster (3–7 months later) antibodies of relatively higher avidity had been produced by these infants.

We found that the DTaP-PRP-T-HB combination vaccine studied produced antibody with BA that correlated closely with total IgG anti-Hib-PS antibody. The critical issue of clinical

relevance is the production of adequate quantities of bactericidal antibody after DTaP–PRP-T–HB combination immunization. Measurement of bactericidal antibody induced by Hib conjugate vaccines is important because of functional differences in idiotypically defined IgG anti–Hib-PS antibody [12]. Antibody avidity did not correlate with bactericidal titers.

Only children with <1.0 μg/mL anti–Hib-PS IgG antibody after the primary series received a CRM_{197}-OS booster. All infants responded to the CRM_{197}-OS booster with a rise in IgG anti–Hib-PS antibody. Seven children showed a rise in anti–Hib-PS IgG antibody level between the primary series and the booster; this rise possibly occurred because of interim exposure to Hib or cross-reacting antigens or the resolution of a developmental delay in the priming process. These 7 children had significantly higher anti–Hib-PS antibody levels and mean AI after boosting than the other boosted children.

Goldblatt et al. [13] recently studied anti–Hib-PS IgG avidity in children receiving whole-cell DTP vaccine (Wellcome, Beckenham, UK) mixed in the same syringe with PRP-T or CRM_{197}-OS Hib conjugate vaccines administered as a 3-dose schedule at 2, 3, and 4 months of age followed by a booster at age 12 months with either of the 2 conjugate vaccines. Similar to our results, they found that anti–Hib-PS IgG antibody avidity rose, while antibody levels fell in the time span between the primary series and the Hib conjugate booster and a differential in postpriming antibody avidity among children with lower (<1 μg/mL) versus higher (≥1 μg/mL) anti–Hib-PS IgG concentrations. Because avidity was lower in vaccinees whose postpriming anti–Hib-PS IgG concentration was <1 μg/mL and these same children had lower postboost anti–Hib-PS antibody levels, Goldblatt et al. suggested that those with lower anti–Hib-PS IgG antibody were not primed. We found 2 children with low anti–Hib-PS antibody levels after boosting, but both had high avidity antibody (58% and 73%). In their study and ours, postboost GM anti–Hib-PS antibody was high even in those with <1.0 μg/mL anti–Hib-PS antibody after the primary series. An Hib conjugate booster in a similar age group of children who were not primed would produce little or no anti–Hib-PS IgG antibody [14]. The difference observed in anti–Hib-PS avidity after boosting in the study by Goldblatt et al. may have been due to the close spacing of primary vaccinations [15]. A broader implication of our work and that of Goldblatt et al. may be that closely spaced vaccinations adversely affects antibody affinity maturation in children. This notion deserves further study.

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CONCISE COMMUNICATIONS

The Induction of Immunologic Memory after Vaccination with *Haemophilus influenzae* Type b Conjugate and Acellular Pertussis–Containing Diphtheria, Tetanus, and Pertussis Vaccine Combination

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The significance of reduced antibody responses to the *Haemophilus influenzae* type b (Hib) component of acellular pertussis–containing combination vaccines (DTaP-Hib) is unclear. A DTaP-Hib vaccine evaluated in infants vaccinated at ages 2, 3, and 4 months showed reduced anti-Hib polysaccharide IgG (geometric mean concentration [GMC], 1.23 μg/mL; 57%, >1.0 μg/mL). Polyribitolribosyl phosphate (PRP) and Hib conjugate (PRP-T) vaccine given as a booster during the second year of life was evaluated for the presence of immunological memory. After boosting, most children achieved anti-PRP IgG >1.0 μg/mL, although the GMC was higher with PRP-T (88.5 μg/mL) than with PRP vaccine (7.86 μg/mL, $P < .001$). The GMC of the PRP group was higher than anticipated for naive PRP recipients of the same age. PRP-specific IgG avidity was significantly higher after boosting than after priming, providing further evidence for the generation of memory. Despite reduced immunogenicity, DTaP-Hib combination vaccines appear to prime for immunologic memory.

An increasing number of countries are changing from whole cell pertussis (wP) to acellular pertussis (aP) vaccines because of reduced reactogenicity. However, unlike wP vaccine combinations, aP-containing diphtheria, tetanus, and *Haemophilus influenzae* type b conjugate (DTaP-Hib) combinations have shown reduced immunogenicity of the Hib component [1]. Although the significance of this for clinical protection is unclear, the US Food and Drug Administration (FDA) recommends that DTaP and Hib vaccines not be given as a combined injection to infants [2].

We conducted a phase II trial of a DTaP-Hib combination vaccine in infants and observed reduced responses to the Hib component. To help evaluate whether vaccinees had been primed for memory, children were randomized in their second year of life to receive either Hib conjugate vaccine or plain Hib polysaccharide polyribitolribosyl phosphate (PRP) vaccine. PRP as an immunogen can be used to mimic natural exposure to Hib and, in young children, can help differentiate booster from primary responses. In addition to IgG levels, we measured the avidity of PRP-specific IgG because an increase can be indicative of the establishment of immunologic memory [3].

Materials and Methods

Study population. Infants eligible for primary immunizations at ages 2, 3, and 4 months with diphtheria-tetanus-pertussis (DTP), Hib, and oral polio vaccines were recruited between June 1996 and January 1997 from general practices in Hertfordshire. Contraindications to further doses were as specified in the national UK guidelines.

Vaccines and immunization schedule. All study vaccines were manufactured by SmithKline Beecham Biologicals, Rixensart, Belgium. The lyophilized Hib tetanus toxoid conjugate vaccine (PRP-T, Hiberix) contained 10 μg of PRP covalently linked to a purified tetanus toxoid (30 μg). The DTaP vaccine (Infanrix) consisted of separately purified pertussis antigens: 25 μg of pertussis toxoid (PT), 25 μg of filamentous hemagglutinin (FHA), 8 μg of pertactin (PRN) with 10 Lf of tetanus toxoid (potency >40 IU/dose) and 25 Lf of diphtheria toxoid (potency specification >30 IU/dose; fiducial limits of batch used 25–56 IU, as determined by the National Institute for Biological Standards and Control, Potters Bar, UK) adsorbed to 0.5 mg of aluminum hydroxide. The PRP-T vaccine was reconstituted with the DTaP vaccine and given by intramuscular injection into the thigh, arm, or buttock.

After reduced Hib antibody responses to the primary immunization course, a booster dose of Hib vaccine was offered to all study participants. Infants were stratified into three groups ac-

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Written informed consent was obtained from parents. The study was approved by the ethics committees in North and East Hertfordshire District, the Institute of Child Health, and Great Ormond Street Hospital for Children National Health Service Trust.

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cording to their postprimary Hib IgG response (<0.15, 0.15–1.0, or >1.0 μg/mL) and were randomized to receive either a single dose of unconjugated PRP vaccine (containing 10 μg of PRP, no adjuvant) or a fourth dose of PRP-T.

Blood samples were obtained before the first dose, 4–6 weeks after the third dose, and before and 4–6 weeks after the booster immunization. Sera were separated, frozen, and stored at −20°C until tested for antibody levels.

Serologic studies. Sera were tested for PRP IgG by a standardized ELISA protocol, as described elsewhere [4]. Antibody concentrations were derived from an international standard preparation and expressed in micrograms per milliliter (lower level of sensitivity, 0.15 μg/mL). PRP IgG avidity was measured by modification of an ELISA incorporating the chaotrope, ammonium thiocyanate [5]. One change to the assay was the use of Hib capsular oligosaccharide conjugated to human albumin (Wyeth Lederle Vaccines and Pediatrics, Rochester, NY) as the solid-phase antigen, in contrast to the PRP-conjugated poly-L-lysine used previously. This change, brought about by the difficulty in obtaining plain PRP, precluded comparison with previously published PRP avidity values because of the lower values obtained with the modified assay. Serum IgG to PT, FHA, and PRN were measured by ELISA, as described elsewhere [6], and expressed in US IgG unitages according to US pertussis reference sera (lot 3 for PT/FHA; lot 4 for PRN).

Statistical evaluation. Antibody levels and avidity indices were log-transformed, and differences in geometric means were compared by regression or Student's *t* test. Differences in proportions were compared by χ^2 or Fisher's exact test. The relationship between PRP IgG levels and antibody levels to the other vaccine components was measured using Spearman's correlation coefficient. Partial correlations between log-transformed antibody levels were calculated, to identify the independent correlations between each pair of antibodies after accounting for their correlation with the other antibodies.

Results

Study subjects. In all, 149 infants (87 boys, 62 girls) were recruited, and 148 received 3 doses of DTaP-Hib vaccine (1 child withdrew from the study after leaving the district). Pre- and postimmunization blood samples were obtained from 144 children, of whom 122 received a booster immunization; pre- and postbooster blood samples were obtained from 120 children (61 PRP, 59 PRP-T). The median ages at first dose and booster immunizations were 8 weeks (range, 7–12) and 16 months (range, 12–21), with no differences between study groups. The median interval between the third dose and the blood sampling was 48 days (range, 28–117) and between the booster dose and the blood sampling, 30 days (range, 27–56).

Immunogenicity. After primary immunization, only 57% of infants achieved a PRP IgG titer of >1.0 μg/mL (table 1), the level considered indicative of long-term protection, compared with 93% of the historical controls given DTwP-Hib vaccines [7]. After boosting, all vaccinees achieved PRP IgG titers above the minimum protective level (0.15 μg/mL), although the proportion achieving titers >1 μg/mL and the geometric mean concentration (GMC) of PRP IgG were higher in those boosted with PRP-T than in those boosted with PRP ($P = .003$ and $P < .001$, respectively; table 1).

In both booster groups, PRP IgG avidity was significantly higher 1 month after boosting than 1 month after completing the primary immunization series (fold increase: PRP group, 2.03, 95% confidence interval [CI], 1.64–2.51; PRP-T group, 1.55, 95% CI, 1.27–1.88). Despite the lower GMCs achieved in the PRP group, the avidity index was higher than in those boosted with PRP-T ($P < .001$; table 1). This difference was still apparent when the results were stratified by PRP IgG levels after the third vaccination ($P < .001$ within each postthird vaccine group; table 2). Within each booster group, the avidity index was not significantly different between those with postthird vaccination levels below or >1 μg/mL (PRP group, $P = .58$; PRP-T group, $P = .14$). The magnitude of the booster response, as measured by fold difference between pre- and postbooster PRP IgG level, was correlated with the fold increase in avidity between postthird and postbooster sera for those boosted with PRP ($r = .36$, $P = .009$), but not for those boosted with PRP-T ($r = .02$, $P = .91$).

Infants with low PRP IgG levels after the third dose had lower antibody titers to all other vaccine antigens (table 2). After adjusting for this individual responsiveness, partial cor-

Table 1. The geometric mean concentration (GMC) of anti–polyribitolribosyl phosphate (PRP) IgG, the proportions of children with antibody titers <0.15 or >1.0 μg/mL, the number with a >4-fold increase in IgG after a booster, and the geometric mean avidity index at age 5 months after primary immunization with 3 doses of acellular pertussis–containing diptheria, tetanus, and *Haemophilus influenzae* type b conjugate (DTaP-Hib) vaccine and immediately prior to and 1 month after a booster dose of Hib conjugate vaccine or plain PRP given at a mean age of 16 months.

		Anti-PRP IgG levels mg/mL				Avidity index, geometric mean (95% CI)
Time measured, group tested	No.	IgG GMC (95% CI)	<0.15 μg/mL *n* (%)	>1.0 μg/mL *n* (%)	>4-fold increase (prebooster:postbooster)	*n*
After primary, all	145	1.23 (0.98–1.58)	7 (4.9)	82 (57)		122	0.05 (0.045–0.056)
Before booster, all	120	0.25 (0.21–0.30)	38 (31.4)	9 (7.5)		ND	
After booster							
PRP	61	7.86 (5.30–11.70)	0	52 (85)	58/61 (95)	61	0.109 (0.091–0.130)
Conjugate	59	88.5 (64.4–121.5)	0	59 (100)	59/59 (100)	59	0.068 (0.058–0.080)

NOTE. CI, confidence interval; ND, not determined.

Table 2. Geometric mean (GM) antibody responses (range) to polyribitoltibosyl phosphate (PRP), diphtheria, tetanus, and pertussis components of combined acellular pertussis–containing diptheria, tetanus, and *Haemophilus influenzae* type b conjugate (DTPa-Hib) vaccine, and Hib response to boosting stratified by postthird vaccination anti-PRP level.

	GM response after third vaccination	
	≤1.0 μg/mL	>1.0 μg/mL
Anti-PRP GM concentration (*n*)	60	60
After PRP boost	2.93 (1.89–4.53)	17.8 (11.2–28.4)
After conjugate boost	54.7 (35.6–84.2)	156 (107–228)
GM avidity index		
After PRP boost	0.104 (0.077–0.141)	0.114 (0.092–0.141)
After conjugate boost	0.061 (0.048–0.078)	0.078 (0.064–0.095)
GM titer of IgG after third vaccination (*n*)	62	82
Diphtheria IU/mL	0.51 (0.43–0.59)	0.94 (0.82–1.06)
Tetanus IU/mL	0.61 (0.52–0.70)	1.35 (1.19–1.53)
Pertussis toxin U/mL	29.95 (25.97–34.58)	46.31 (40.96–52.35)
PRN U/mL	57.02 (45.61–71.26)	114.7 (96.41–136.45)
Filamentous hemagglutinin U/mL	79.4 (68.15–92.51)	93.1 (79.3–109.3)

relation analysis revealed the strongest independent positive correlation between the Hib and tetanus antibody levels (r = .23, $P < .05$). Despite the lower than specified diphtheria potency, all vaccinees had antibody levels >0.05 IU/mL after the third vaccine dose.

Discussion

Recent studies describing the reduced immunogenicity of the Hib component in DTaP-Hib combination vaccines [1, 8, 9], together with an increasing awareness of the importance of the induction of immunologic memory for long-term protection against Hib disease, have highlighted the need for sensitive ways to assess immunologic memory. Analyses of the antibody levels achieved after plain PRP or Hib conjugate vaccine boosters in primed infants have been used for this purpose, but interpretation of data has been hampered by the ethical constraints of generating contemporary control data from unprimed age-matched children given a single dose of plain or conjugated PRP. Historical comparisons may be informative but require careful review of the literature. For example, in a recent study of children with reduced primary responses to a combination vaccine [9], a PRP IgG GMC of 9.02 μg/mL after a booster dose of a Hib conjugate vaccine was considered indicative of a memory response despite higher values in the literature for a single dose of the same vaccine in unprimed 15-month-old children [10].

Our study is the first to directly compare PRP IgG levels induced by plain PRP or conjugated Hib booster in recipients of DTaP-Hib and to use the measurement of PRP IgG avidity to aid interpretation of the results. PRP is a plain polysaccharide. It can stimulate memory cells but elicits poor responses from naive B cells in children <2 years old. Thus, it is better than the highly immunogenic conjugate vaccines for assessing the presence of immunologic memory. An antibody titer >1 μg/mL is rarely achieved by toddlers receiving plain PRP for the first time in the second year of life [11]. In our study, the GMC achieved by the group boosted with plain PRP (7.86 μg/mL) indicates that immunologic memory was established.

Antibody avidity increases over time and after boosting in persons successfully primed for a memory response and has been used as a surrogate marker for the induction of memory to the Hib component of the DTwP-Hib conjugate [5] and of the pneumococcal capsular polysaccharides contained in pneumococcal conjugate vaccines [12]. In this study, PRP IgG avidity was greater after boosting than after primary immunization, providing additional evidence of the successful generation of immunologic memory by the DTaP-Hib vaccine, even in those with poor postthird PRP vaccine IgG responses. Of interest, the postbooster avidity index in the 3 infants with postthird vaccination IgG PRP levels <0.15 μg/mL who received a PRP booster (0.081, 0.097, and 0.078 μg/mL, respectively) was higher than that observed after primary immunization, although their postbooster PRP IgG levels increased only modestly (0.38, 0.69, and 2.63 μg/mL, respectively). Thus, T cell help and the generation of immunologic memory may occur in the absence of a detectable primary antibody response.

Children with reduced PRP IgG responses also had significantly reduced antibody responses to all vaccine antigens, but most markedly to the tetanus component. This finding supports the assertion of Dagan et al. [13], that dominance of carrier-specific B cells (also known as epitopic suppression) is unlikely to be the major mechanism responsible for the reduced Hib response. The general correlation between antibody responses to all vaccine antigens is also seen with DTwP-Hib vaccines in UK infants (Miller E, personal communication) and may reflect individual variation in the degree of age-dependent maturation of the immune system.

The rationale for developing aP vaccines was to reduce reactogenicity. However, the benefit of an improved reactogenicity profile with DTaP-Hib vaccines needs to be balanced against the possible disadvantages of a reduced Hib response or an

increased number of injections if, as recommended by the FDA, such combinations are not used in infants. Our study suggests that DTaP-Hib combination vaccines adequately prime for memory despite reduced Hib responses. The protective capacity of such combinations has not been studied, but the PRP IgG titers achieved after primary immunization with DTaP-Hib vaccines were higher than those seen with PRP–diphtheria toxoid conjugate vaccines, which have been highly effective in Finland [14]. In populations at high risk of early onset Hib disease [15], DTaP-Hib combination vaccines might not be appropriate, as the reduced levels of antibody in the period between priming and boosting may be important in determining protection. Avidity measures and booster responses are at best surrogates of protection, and careful postlicensure disease surveillance in countries switching to DTaP-Hib combination vaccines will be essential, particularly if no booster is given in the second year of life.

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SUPPLEMENT ARTICLE

Combination Vaccines: Defining and Addressing Current Safety Concerns

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Combination vaccines have been in use for >50 years. Historical problems with vaccines, including intussusception after rotavirus vaccine, carrier suppression with tetanus toxoid conjugate vaccines, and decreased immunogenicity of some *Haemophilus influenzae* type b conjugate vaccines when mixed with acellular pertussis-diphtheria-tetanus, have contributed to some misperceptions about current vaccines. There is no evidence that adding additional vaccines through combination products increases the burden on the immune system, which has the capability of responding to many millions of antigens. Combining antigens usually does not increase adverse effects—in fact, it can lead to an overall reduction in adverse events. Combination products simplify immunization and allow for the introduction of new vaccines without requiring the vaccinee to make additional visits to his or her health care provider. Licensed combination vaccines undergo extensive testing before approval by the United States Food and Drug Administration to assure that the new products are safe and effective.

Combination vaccines that have been in widespread use for >50 years in the United States and other countries have brought enormous benefits to children. Several products not yet available in the United States combine antigens to induce protection against 4–5 different diseases into 1 injection (table 1). Compared with components administered separately, combination products decrease the number of injections, amount of pain, and cumulative exposure to preservatives and stabilizers that can contribute to adverse events [1, 2]. Moreover, combination products have simplified immunization delivery, allowing for the introduction of additional vaccines to the immunization schedule without increasing the number of clinic visits [3]. Without combination products, children would need 68 or 69 injections by 6 years of age to receive the recommended doses of currently recommended vaccines (table 2). We now administer these 68 or 69 doses of vaccines with only 18–23 injections, depending on which combination products are used.

At least 100 additional vaccines are under development [4]; some of these vaccines are likely to be fully developed and introduced into the routine immunization schedule. If the numbers of vaccines recommended for routine use continues to expand, at some point there could be a limit to the number of injections and antigens that we can administer at one time, and we may need to consider adding additional visits to the schedule. Also, in order to protect children against severe diseases that occur early in life, such as respiratory syncytial virus, we may need to administer the new vaccines at more frequent intervals (e.g., monthly) during the first few months of life. The public health community has resisted adding visits because the increased cost and inconvenience could have a negative impact on immunization coverage rates.

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Table 1. Combination vaccines currently licensed in 1 or more countries.

DTP/DTaP
Inactivated poliovirus vaccine
Oral poliovirus vaccine
Measles, mumps, and rubella
Mumps and rubella
Meningococcal A, C, Y, W135
Pneumococcal polysaccharide
Pneumococcal conjugate (PCV7)
DTP-Hib
DTaP-Hib
DTP-Hep B
DTP-Hep B-Hib
DTP-IPV
DTaP-IPV-Hib
DTaP-Hep B-IPV

NOTE. DTaP, acellular pertussis-diphtheria-tetanus; DTP, diphtheria-tetanus-pertussis; Hep B, hepatitis B; Hib, *Haemophilus influenzae* type b; IPV, inactivated poliovirus vaccine.

CONCERNS AND MISPERCEPTIONS ABOUT COMBINATION VACCINES

Recent publicity about new combination products has led to the misperception that combination vaccines are new. Most combination products are not new; diphtheria-tetanus-pertussis (DTP) has been in use for >50 years in this country, oral poliovirus vaccine was used for 38 years, and the measles, mumps, and rubella (MMR) vaccine has been available for 28 years.

Other concerns expressed recently include the possibility that (1) too many vaccines might overload the immune system, (2) combination vaccines may be less effective than vaccines administered separately, and (3) combination vaccines can cause a greater number of adverse reactions than do vaccines administered separately (table 3). Some recent surveys indicate that many doctors, parents, and pediatricians in training are concerned about the possibility of increased side effects and decreased immunogenecity when adding new vaccines at regularly scheduled visits (table 4) [5]. What is the origin of these concerns and misperceptions about combination vaccines?

Concerns about decreased effectiveness. Concern regarding decreased effectiveness when vaccines are combined has come from studies indicating that the response to *Haemophilus influenzae* type b (Hib) conjugate vaccine can be decreased when combined with acellular pertussis-diphtheria-tetanus (DTaP) and the failure of these combination products to be approved by the US Food and Drug Administration [6, 7]. These issues have been addressed by others; my opinion is that slightly lower geometric mean anti–polyribosyl ribitol phosphate (PRP) concentrations observed when some of these products are combined will not translate into a decreased effectiveness of these vaccines, but a greater understanding of the cause for lower responses is needed [8]. The primary criterion for rejecting a combination product should be decreased percentages of children who develop protective antibody concentrations. Even when decreased seroconversion rates were noted for specific lots of PRP-OMP (*Neisseria meningitidis* outer membrane protein conjugate vaccine), high protection was observed, which indicated that immunologic priming had occurred [9]. New guidelines are indicated in this area, and some flexibility should be provided because the issues may differ by type of product. The US Food and Drug Administration has difficult decisions to make, but some variability in geometric mean antibody concentrations is to be expected and should not preclude the approval of new combination products.

Sources of unfounded concerns about adverse events. Concerns regarding the possibility of increased adverse events by adding vaccines together are unfounded for such vaccines as MMR. The rates of fever, rash, or both in the 3 weeks after MMR vaccination are similar to the rates when measles vaccine alone is administered; the inclusion of rubella vaccine adds a variable incidence of arthralgia, depending on the age and sex of the vaccinee [10, 11]. The overall incidence of adverse effects from the combination product is no greater than the sum of the rates that would occur were each of the products to be administered separately.

The incidence of fever, redness, and swelling at the injection site after tetanus toxoids and diphtheria vary according to age, dose, previous number of immunizations, and prevaccination antibody concentration [12]. Also, aluminum absorbed prod-

Table 2. Vaccine doses and injections recommended for children birth to 6 years of age, as of May 2001.

Vaccine	No. of doses
Diphtheria	5
Tetanus	5
Pertussis	5
Hepatitis B	3
Measles	2
Mumps	2
Rubella	2
Haemophilus influenzae type b	3–4
Inactivated poliovirus vaccine (3 × 4)	12
Varicella	1
Pneumococcal conjugate (PCV7; 7 × 4)	28
Total doses	68–69
No. of injections	18–23

NOTE. From the Committee on Infectious Diseases [3].

Table 3. Misperceptions about combination vaccines.

Combination vaccines are new.
Children receive too many vaccines.
Immune system "overload."
Combined vaccines are less effective.
Combined vaccines cause more reactions.

ucts produce higher local reaction rates than do nonadsorbed products. Combining diphtheria toxoid with tetanus toxoid results in higher reaction rates than tetanus toxoid alone, but not higher than diphtheria toxoid alone. The overall effect of combination products is a reduction in adverse events, compared with administering these products separately. Some new products that combine DTaP with hepatitis B or Hib vaccine cause slightly higher rates of local erythema than does either product administered separately (figure 1), but the incidence is not greater than the cumulative effect of the 2 products administered separately, and other studies found no differences [6, 13]. We need new data collection and analytic methods to demonstrate this quantitatively.

When whole-cell DTP was mixed with inactivated poliovirus vaccine at the time of administration by means of a dual-chambered syringe, the combined product was associated with a decreased incidence of local reactions, compared with DTP administered alone (figure 2) [14]. I believe that this phenomenon was most likely the result of diluting the whole-cell DTP into a 1-mL dose rather than the usual 0.5 mL. The less concentrated components could result in decreased local irritation. As we continue to combine additional products into single injections, consideration should be given to increasing the volume to 1 mL, which is easily tolerated by children and could be associated with decreased reactions.

LESSONS FROM RHESUS ROTAVIRUS VACCINE (RRV)

RRV is a combination of 4 live viral vaccines. The withdrawal of RRV in the United States because of a demonstrated causal association with intussusception has undoubtedly contributed to current concerns about the safety of new vaccines. We do not yet understand the factors contributing to the cause of this complication, and we do not know if modifications in this vaccine or other rotavirus vaccines will decrease or eliminate the risk of intussusception. The experience with RRV provides reassurance that an effective system is now in place to monitor adverse events and to investigate the possibility of serious complications from vaccines. The early recognition of a possible problem, prompt and efficient investigation, and effective actions taken by the manufacturer, the Centers for Disease Control and Prevention, and the American Academy of Pediatrics should increase confidence that concerned officials are monitoring the safety of new vaccines [15–17]. If extensive testing of new rotavirus vaccines reveals no increased risk of intussusception, then there will be a need for intensive educational programs to inform the public about the safety of these new products.

The experience with rotavirus vaccine has led to suggestions that safety testing of new vaccines be modified to include extended prelicensure safety trials after demonstration that a vaccine is efficacious [18]. Extended prelicensure assessment in controlled trials of the recently approved pneumococcal conjugate vaccine in California and among Native Americans has provided an example of how some expanded safety data might be generated. After data became available that demonstrated that the vaccine was efficacious, the investigators maintained the double-blind study protocol and collected extended safety and efficacy data for many months, thus collecting information about duration of protection from the vaccine and adverse events during a longer period of time [19]. Moreover, other randomized trials have been continued, and they have generated more data on safety than might have been accrued if the investigators had been obligated to give placebo recipients pneumococcal vaccine as soon as efficacy was demonstrated in the first study [20]. The extended safety testing of this vaccine should provide increased reassurance of the safety of this vaccine.

Some experts have argued that new vaccines should have a limited licensure to restrict use of the new vaccines pending additional safety data. I do not support this argument; the potential benefits from a new vaccine would be unnecessarily denied to a large segment of the population.

Questions have been raised regarding whether or not RRV should have been recommended for universal use after studying ~10,000 vaccine recipients. The Advisory Committee on Immunization Practices and the Committee on Infectious Diseases of the American Academy of Pediatrics debated this issue, and some committee members expressed concern about going from a database of 10,000 to recommending the vaccine for 4,000,000 children a year. There appeared to be no alternative available to the committees, because no high-risk groups could be identified, and more limited recommendations would have precluded third-party payers from compensating doctors and parents for the vaccine. Alternatives should be explored with selected new types of vaccines to have permissive, rather than "recommended," guidelines for brief periods of time (e.g., 6 months to 2 years), which would allow for collection of more data before making recommendations for universal use.

This approach would not be appropriate for some new vaccines, such as the pneumococcal conjugate vaccine, because this vaccine has enormous potential for preventing pneumonia, meningitis, and other severe disease due to *Streptococcus pneu-*

moniae. Withholding this vaccine from young children could not be justified. These decisions are complex and require evaluation of the risks of disease, severity of complications, availability of alternative approaches to disease prevention, and economic factors that are weighed by advisory committees before making recommendations.

Do vaccines increase the burden on the immune system? Several parents' groups and some doctors have been concerned that the administration of several antigens at one time might have an adverse affect on the host immune system. What is the origin of this concern? Dagan et al. [21] have shown that large doses of tetanus toxoid administered simultaneously with a pneumococcal conjugate vaccine, using tetanus toxoid as the carrier protein, resulted in a dose-dependent decreased response to PRP, which was administered as a PRP-tetanus combination (figure 3) [22]. The recently licensed pneumococcal conjugate vaccine uses a different carrier protein (modified diphtheria toxoid), and there is no evidence of carrier suppression with this vaccine. Nevertheless, the observation that carrier suppression can occur contributes to the concern about administration of large doses of some antigens, possibly suppressing the host immune response to other antigens [22].

A second phenomenon that may contribute to the concern about immune suppression is transient decreased delayed-type hypersensitivity to skin test antigens after MMR vaccines [23–25]. Each of these vaccines, when administered separately, is associated with decreased skin test reactivity for a few weeks. The combined MMR vaccine is also associated with a transient decline in delayed-type hypersensitivity, but the decline is not of greater magnitude or greater duration than that of any of the 3 vaccines administered separately (figure 4). Therefore, the combination product is associated with modification of the immune system for a shorter duration of time than if the products were administered separately.

High-titer measles vaccines. In the late 1980s, efforts were undertaken to induce protection against measles in children aged <1 year by administering measles vaccines of increased

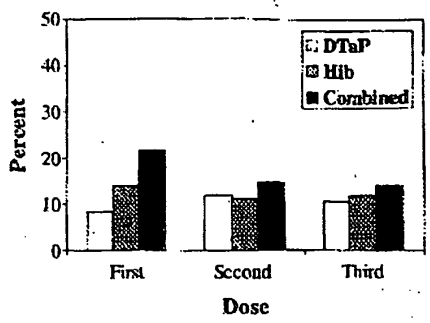

Figure 1. Erythema after combined or separate administration of acellular pertussis-diphtheria-tetanus (DTaP) and *Haemophilus influenzae* type b (Hib) vaccines. From Pichichero et al. [6].

titer [26]. Safety testing revealed no increase in the rates of fever or rash, with the high-titer vaccines containing $>10^3$ cfu per milliliter, but long-term follow-up revealed an increased risk of delayed mortality in girls residing in developing countries where baseline infant mortality rates were >100 deaths per 1000 live births [27–29]. Testing of lymphocyte function by skin testing and other assays revealed a small, but statistically significant, lower response in children who had received high-titer vaccine, compared with children who received standard- or medium-titer vaccines when the children were tested 12–18 months after immunization [30]. The increased mortality rates in girls were seen only with vaccines that contained 100-fold increases in vaccine titer ($\geq 10^5$ cfu/dose), not with vaccines that contained 10-fold increases in titer (10^4–$10^{4.9}$ cfu/dose). Therefore, there appears to be a significant safety margin with the current measles vaccines that contain 10^3–$10^{3.5}$ cfu/dose. Moreover, this phenomenon is most likely limited to measles vaccines, because measles virus is known to be associated with direct effects on the immune system [31]. Also, the increased mortality was noted only in countries where the infant mortality rate was >100 deaths per 1000 live births; no increase in

Table 4. Factors affecting parent and physician concerns about multiple injections.

Factors influencing concerns about multiple injections	Practicing physicians (n = 213)	Parents[a] (n = 197)	Residents[a] (n = 74)
Pain of multiple injections to child	82	62[b]	67
Possibility of increased side effects	55	55	45
Possibility of reduced immunogenicity	42	51	45
Negative effect on patient return	56	—	55
Negative effect on office staff	52	—	33[c]

NOTE. From Woodin et al. [5].

[a] Parents and residents compared with practicing physicians.

[b] $P<.001$, by χ^2 test.

[c] $P=.006$, by χ^2 test

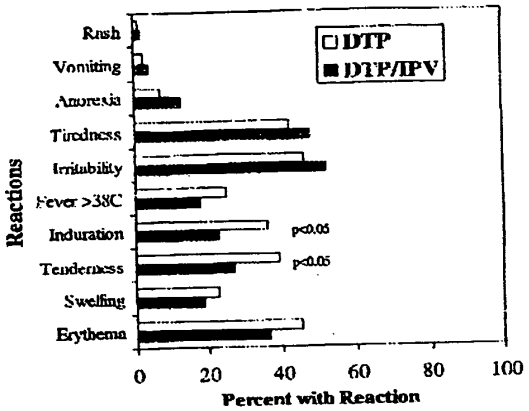

Figure 2. Adverse events after vaccination with diphtheria-tetanus-pertussis (DTP) and inactivated poliovirus vaccine (IPV) in a dual-chambered syringe compared with DTP alone at 6 months of age. From Halsey et al. [14].

mortality was associated with high-titer vaccine in developing countries with infant mortality rates of 40–90 per 1000 live births or in more developed countries. I believe that the adverse effect from high-titer vaccines was associated with increased exposure to multiple other infections that children in impoverished developing country populations encounter early in life, along with possible contributions from underlying malnutrition [29]. There is currently no satisfactory explanation as to why the phenomenon was observed in girls only, but there are other immune-based disorders, such as autoimmune diseases, that occur more frequently in girls than they do in boys.

Children are exposed to many viruses and bacteria. The development of even a mild viral upper respiratory infection can lead to immune responses to 4–10 different antigens; children who develop streptococcal pharyngitis can develop immune responses to 25–50 different bacterial antigens, and a single bacteria strain colonizing the gastrointestinal tract can lead to immune responses to 15–50 antigens [32–33]. Newborn infants became colonized with numerous bacteria in the intestinal and respiratory tracts within a few hours of life. Some recently developed vaccines protect children against disease by use of only 1 or 2 antigens. For example, Hib conjugate vaccines use only the PRP antigen plus a carrier protein. Children with invasive disease due to *Haemophilus influenzae* are exposed to at least 10 times as many antigens. Hepatitis B vaccine consists of only a single antigen, but infection with hepatitis B can lead to immune responses to 4 different antigens. Thus, giving children vaccines that protect against infectious diseases can actually reduce the number f antigens to which the immune system is required to respond. Although the route of exposure f r vaccines is usually parenteral, as compared with mucus membrane exposure for natural exposure to the infectious agents, there is no evidence that parenteral exposure would lead to substantially different safety effects if the dose is controlled.

Some experts have estimated that the human immune system is capable of responding to at least 10 million different antigens [34]. It is unlikely that we will use up the ability of the immune system to respond to multiple antigens in vaccines in the foreseeable future. The US Army has administered multiple vaccines to military recruits for many years. A study of 99 men who received up to 134 mL of multiple vaccines and 6–93 skin test microbial antigens revealed no harmful affects after 25 years [35]. The immune system of healthy adults differs from that of infants, but this experience provides some reassurance regarding the lack of harmful effects from administering much larger doses of antigens than are usually administered.

Unfounded concerns about varicella vaccine. Another source of possible concern about simultaneous administration of multiple antigens surfaced when some children developed varicella-like rashes within a few weeks after receipt of varicella vaccine, which was often administered simultaneously with MMR. The vaccine manufacturer set up a study to have viral cultures obtained from children or adults with unusual illnesses after vaccination. Genetic sequencing of the isolated viruses revealed that the vast majority of isolates obtained in the first 2 weeks after varicella vaccine were caused by wild-type virus in children who had been exposed before vaccination. Almost all isolates obtained from people with ≥300 skin lesions within 2 weeks after vaccination were caused by the wild-type viruses associated with unrecognized exposures to varicella-zoster virus infections [36]. If the vaccine is administered late in the incubation period, the vaccine does not protect against varicella, and children will develop disease shortly after receiving the vaccine. There is no evidence of any increased rates of reactivity from varicella vaccine when it is administered simultaneously with MMR [37].

CONCERNS ABOUT AUTISM AND VACCINES

In the United Kingdom, concern about the possible association between MMR vaccines and autism has led to a decline in the

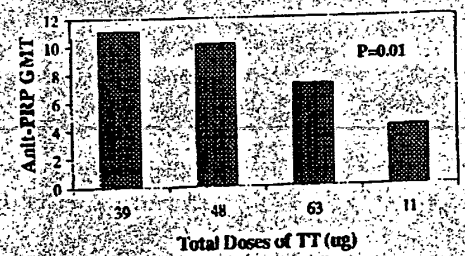

Figure 3. Response to polyribosyl ribitol phosphate (PRP) when administered with vaccines sharing common protein epitopes. GMT, geometric mean titer; TT, tetanus toxoid. From Dagan et al. [21].

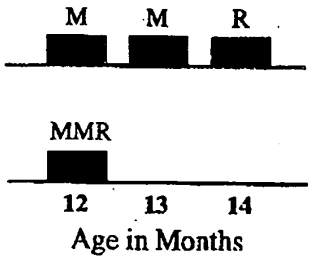

Figure 4. Decreased delayed-type hypersensitivity after administration of measles, mumps, and rubella (MMR) vaccines, separately or in combination.

acceptance of MMR from 90% in 1994 to 75% in mid-1999. These concerns have been generated primarily by one group of investigators at the Royal Free Hospital, London, under the direction of Dr. Andrew Wakefield [38], who hypothesized that the simultaneous administration of MMR vaccines somehow contributes to an increased risk of autism. Wakefield found evidence of measles antigen in the intestinal wall of people with inflammatory bowel disease by means of immunofluorescence assay. Subsequently, several other centers around the world have failed to confirm these findings, and Iizuka et al. [39] demonstrated that the monoclonal antibody used by Wakefield cross-reacted with human tissue and that positive staining cells were not unique to Crohn's disease. Wakefield subsequently noted that 12 children with inflammatory bowel disease also had pervasive developmental disorders commonly known as autism. The parents of 8 of these children believed that some of the symptoms of autism had developed within the few months after administration of MMR vaccine. Wakefield hypothesized that the simultaneous administration of MMR antigens could contribute to autism by contributing to the development of intestinal inflammation and absorption of toxins [40]. Subsequent studies by an interdisciplinary group of scientists who used a novel case series approach have demonstrated no epidemiologic evidence for a causal association between MMR and autism [41]. There was no step up in the incidence of autism after the introduction of MMR in 1989 in the United Kingdom; there is no difference in the age at onset of autism for children before and after the introduction of MMR; there is no temporal association between MMR and autism; and there is no new unique syndrome of this type of disorder after MMR.

An expert panel convened by the American Academy of Pediatrics conducted a detailed review of this subject and concluded that the available evidence does not support the hypothesis that MMR vaccine causes autism, associated disorders, or inflammatory bowel disease, and that separate administration of MMR vaccines t children provides no benefit over administration of the combination MMR vaccine [42]. Concerned parents' groups in the United States are lobbying for more research on autism [43, 44]. Identifying the true pathogenesis of this disorder should help resolve this and related controversies.

In conclusion, we need to emphasize that children are healthier today than they ever have been in the past because of the use of many vaccines that effectively prevent illnesses. The use of combination products decreases the number of injections children receive, and future combination products will allow us to protect children against additional illnesses without adding immunization visits or increasing the number of injections children receive.

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